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ORIGINAL ARTICLE

Involvement of *AtLAC15* in lignin synthesis in seeds and in root elongation of *Arabidopsis*

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Abstract Laccase, EC 1.10.3.2 or *p*-diphenol:dioxygen oxidoreductase, has been proposed to be involved in lignin synthesis in plants based on its in vitro enzymatic activity and a close correlation with the lignification process in plants. Despite many years of research, genetic evidence for the role of laccase in lignin synthesis is still missing. By screening mutants available for the annotated laccase gene family in *Arabidopsis*, we identified two mutants for a single laccase gene, *AtLAC15* (At5g48100) with a pale brown or yellow seed coat which resembled the *transparent testa* (*tt*) mutant phenotype. A chemical component analysis revealed that the mutant seeds had nearly a 30% decrease in extractable lignin content and a 59% increase in soluble proanthocyanidin or condensed tannin compared with wild-type seeds. In an in vitro enzyme assay, the developing mutant seeds showed a significant reduction in polymerization activity of coniferyl alcohol in the absence of H_2O_2. Among the dimers formed in the in vitro assay using developing wild-type seeds, 23% of the linkages were β-O-4 which resembles the major linkages formed in native lignin. The evidence strongly supports that *AtLAC15* is involved in lignin synthesis in plants. To our knowledge, this is the first genetic evidence for the role of laccase in lignin synthesis. Changes in seed coat permeability, seed germination and root elongation were also observed in the mutant.

Keywords Laccase · Lignin synthesis · Proanthocyanidin metabolism · *Arabidopsis*

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Introduction

Laccases, EC 1.10.3.2 or *p*-diphenol:dioxygen oxidoreductases, are members of a larger group of proteins called the multicopper-containing enzymes including ascorbate oxidases and ceruloplasmins. Laccase was first discovered in 1883 in the sap of the Japanese lacquer tree (Yoshida 1883) and is now found to be widely distributed in plants and fungi (Mayer and Staples 2002). The fungus laccases are so far the most extensively studied and are known for their roles in lignin degradation, bioremediation and many other applications (reviewed in Mayer and Staples 2002). Despite many years of research, the physiological function of laccase in plants remains largely unknown. Based on their in vitro activities and gene expression, laccases in plants are reputedly involved in lignin synthesis (Sterjiades et al. 1992; Bao et al. 1993), wound healing (McCaig et al. 2005), iron acquisition (Hoopes and Dean 2004), response to stress (Liang et al. 2006) and maintenance of cell wall structure and integrity (Ranocha et al. 2002).

Gene expression analysis and immunolocalization of laccases indicated a close association of laccase with

the lignifying process in vascular tissues (e.g. LaFayette et al. 1999; Sato et al. 2001). Purified laccase enzymes polymerized monolignols, the precursors of lignin (Sterjiades et al. 1992; Bao et al. 1993). However, despite many years of effort, there is no genetic evidence for laccase involvement in lignin synthesis. Overexpression of a poplar laccase caused a dwarf phenotype in transgenic tobacco plants; however, a change in the lignin content of the transgenic plants has not been demonstrated (Dean et al. 1998). Down-regulation of expression of a laccase gene, *lac3*, using an antisense approach in poplar (*Populus trichocarpa*) tree also did not alter lignin content (Ranocha et al. 2002).

Laccases comprise a multi-gene family in all plants studied. *Arabidopsis* has 17 annotated laccase genes (www.arabidopsis.org) (McCaig et al. 2005). The wide-spread genetic redundancy of laccase in plants may have made it difficult to reveal any phenotype related to lignification. A defect in a single laccase can easily be compensated for by other laccase genes. Alternatively, different laccases may play different roles in plants. The laccase genes used in previous studies may not be involved in lignin synthesis at all. To address these two possibilities, we have taken a reverse genetics approach to systemically target and disrupt all the laccase genes in *Arabidopsis*. By examining T-DNA insertional mutants that are available for *Arabidopsis* laccase genes, we uncovered two mutants for the laccase gene *AtLAC15* (At5g48100), with altered seed coat color. It is known that mutant seeds in *Arabidopsis* defective in proanthocyanidin (PA) or condensed tannin synthesis often appear yellow or pale brown (Shirley et al. 1992, 1995; Chapple et al. 1994; Debeaujon et al. 2001). In fact, a nearly complete pathway of PA synthesis was elegantly revealed by taking advantage of the unique phenotype of the mutants (reviewed in Chapple et al. 1994; Shirley et al. 1995; Debeaujon et al. 2003). In the last step of the PA synthesis pathway, it has been proposed that the oxidation of PA, presumably by peroxidase or polyphenol oxidase, produces brown end-products which contribute to the dark brown seed color (Debeaujon et al. 2000, 2003; Kitamura et al. 2004). Considering the seed color phenotype and the proposed role of laccase in lignification, we thus examined PA and lignin contents in *lac15* seeds and found that the mutation in *AtLAC15* caused an accumulation of soluble PA and a reduction in extractable lignin content in the seeds. In a recent study, Pourcel et al. (2005) demonstrated that *AtLAC15* is involved in the oxidative polymerization of flavonols including epicatechin in an in vitro assay and thus in PA metabolism. Here we provide evidence for the role of *AtLAC15* in lignin synthesis or polymerization of monolignols in *Arabidopsis* seeds and the involvement of *AtLAC15* in root elongation.

Materials and methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (Columbia 0 ecotype) were sown in a well-watered potting mix (Enriched Potting Mix, Miracle-Gro Lawn Products, Inc., Marysville, OH), and kept in a cold room (4°C) for 2–3 days. Seeds were germinated and seedlings were grown on a light shelf under a 14/10 h light/dark photoperiod. Light was supplied by four cool-white florescent bulbs, reaching an intensity of approximately 120 μmol m^{-2} s^{-1} on the surface of the shelf. Plant tissues were harvested at various times for analysis. To harvest seeds, plants were moved to a greenhouse after bolting. Once the bottom siliques started to turn yellow, the inflorescence stems were bagged in pollination bags (Seedboro Equipment Co., Chicago, IL). Seeds were harvested after all siliques in the bag had become yellow and dry. After harvesting, seeds were capped in 15 ml centrifuge tubes that were wrapped with aluminum foil and stored at room temperature. The light in the greenhouse was supplied by three 1,000 W E25 HPS light bulbs in conjunction with sunlight during the winter, reaching a light intensity of approximately 150 μmol m^{-2} s^{-1} on the surface of the bench. The temperature in the greenhouse was less stable than in the growth room, varying from 21 to 27°C depending on the season of the year.

The T-DNA insertional mutant lines of laccase At5g48100, SALK_002972 (*tt10-4/lac15-1*) and SALK_128292 (*tt10-2/lac15-2*) in the Columbia 0 ecotype background (Alonso et al. 2003), were obtained from the ABRC stock center at Ohio State University. Insertional mutant information was obtained from the SIGnAL website at http://www.signal.salk.edu and verified by PCR and RT-PCR methods.

Plasmid constructs and analyses of transgenic plants

The promoter (1.5 kb before 5′UTR) of *AtLAC15* was PCR-amplified from *Arabidopsis* genomic DNA using a 5′-upstream primer (5′-GTTAATTAACCAAGAA TCGATGAGCGG-3′) and a 3′-downstream primer (5′-AGGCGCGCCGCATCATCTTCTTGGTCCG-3′) and cloned into the Zero Blunt PCR Cloning vector (Invitrogen, Carlsbad, CA). All PCR amplifications were carried out with high-fidelity DNA polymerase

(PfuUltra DNA polymerase, Stratagene, La Jolla, CA). The cloned promoter was verified by DNA sequencing and subcloned into a pBI121 binary vector to drive expression of a GUS gene ($P_{laccase}$::GUS). Plants were transformed with *A. tumefaciens* (LBA4404) using the floral dipping method (Clough and Bent 1998). The transformants were selected on plates containing 50 μg/ml kanamycin and were verified using a PCR method.

GUS staining for promoter analysis

T1 and T2 transgenic plants carrying the $P_{laccase}$::GUS construct were assayed for a GUS color reaction following a method described by Stangeland and Salehian (2002). Tissues from plants at various developmental stages were collected for GUS analysis.

The butanol–HCl method for proanthocyanidin quantification

The soluble PA content was determined using a method described by Mole and Waterman (1987a) and Xie et al. (2003). To extract soluble PA, approximately 100 mg of seeds were ground and extracted in 10 ml 70% aqueous acetone containing 5.26 mM fresh sodium metabisulphite in polypropylene tubes for 1 h at room temperature. The mixture was centrifuged at 10,000 g for 5 min, and the supernatant was collected. The seed residues were extracted two more times as described above. After all the extract was combined, three 1 ml aliquots were taken and each was mixed with 0.5 ml of a butanol/HCl mixture (95% butan-1-ol and 5% concentrated HCl) in polypropylene tubes. The tubes were heated at 95°C for 1 h and cooled to room temperature. The absorbance of the solution was determined at 550 nm. Unheated blanks were prepared in an identical manner and values were subtracted to correct for background absorbance. In this method, the HCl catalyzes depolymerization of condensed tannins as an endo-type reaction in butanol to produce a red anthocyanidin product that can be detected spectrophotometrically. To calculate the relative amount of soluble PA, a standard curve was first constructed using the absorbance at 550 nm of soluble PA extracted from different amounts of mutant seeds. Absorbance at 550 nm of soluble PA from each sample was converted into seed weight based on the standard curve, and the weight was then normalized by the amount of seeds (weight) in each sample. The experiment was conducted three times using independent seed batches, and each sample was assayed as triplicates to obtain a mean value.

Extractable lignin content

To determine extractable lignin content, the thioglycolic acid (TGA) assay was carried out according to the protocol outlined by Campbell and Ellis (1992) with slight modifications. After the seeds were ground in liquid nitrogen, ~50 mg powder was extracted with 70% ethanol until there was no absorbance at 280 nm. The residue was then extracted with DMSO for 12 h at room temperature; the extraction was repeated two more times until the samples were starch-free (Fry 1988). The cell wall residues were washed six times with 70% ethanol to remove DMSO and finally washed two times with 100% acetone. The cell wall residues were collected by centrifugation at 16,000 g for 10 min and were air-dried. After 1 ml absolute methanol was added to the wall residues, the tube was capped, mixed to resuspend the pellet and incubated at 80°C for 2 h**.** Insoluble material was collected by centrifugation at 10,000 g for 5 min. The pellet was resuspended in 1 ml distilled water by vortexing. Insoluble material was collected by centrifugation at 10,000 g for 5 min. The pellet was then mixed with 750 μl distilled water, 250 μl concentrated HCl and 100 μl thioglycolic acid. The tube was capped, mixed and incubated at 80°C for 3 h. The insoluble material was collected by centrifugation at 16,000 g for 15 min, and the supernatant was discarded. The pellet was resuspended in 1 ml distilled water and recollected by centrifugation at 16,000 g for 15 min. The resulting pellet was resuspended in 1 ml 1 M NaOH. The capped tubes were gently agitated for 12 h at room temperature. Insoluble material was collected by centrifugation at 10,000 g for 5 min and discarded. The supernatant was transferred to a fresh 2 ml microfuge tube and mixed with 200 μl concentrated HCl. The tubes were capped, shaken vigorously for 5 s, and incubated for 4 h at 4°C. The resulting precipitate was collected by centrifugation at 10,000 g for 5 min. The pellet was dissolved in 1 ml NaOH. After a 50-fold dilution with 1 M NaOH, the absorbance of the samples at 280 nm was recorded. The experiment was conducted three times using seeds from independent batches, and each sample was assayed as triplicates to obtain a mean value. Lignin content was calculated based on a standard curve of beech lignin (Aldrich, Milwaukee, WI), which was assayed under the same conditions as the samples.

In vitro monolignol polymerization assay

The in vitro monolignol polymerization assay was modified from Sterjiades et al. (1992). Developing seeds (stages 4–5 in Fig. 1b) were carefully separated from

seed pods in Petri dishes containing distilled water that were placed on ice. The seeds were then carefully transferred into microfuge tubes and weighed for fresh weight after removing residual water. Approximately 10 mg of seeds were incubated in 200 µl 150 mM potassium phosphate buffer (pH 6.8) containing 75 units catalase (Calbiochem, San Diego, CA) for 1 h at 37°C to remove endogenous H_2O_2 and therefore prevent peroxidase activity (Pourcel et al. 2005). Fifty microliters of 10 mM coniferyl alcohol (Sigma, St. Louis, MO) was then added to the reaction mixture and the tubes were incubated at 37°C for 16 h. For the control samples, no substrates were added to the seeds after catalase treatment. The reaction mixture was separated from seeds by centrifugation and used for Liquid Chromatography-Mass Spectrometry (LC–MS) analysis.

LC–MS was performed using a LCQ (Thermo Finnigan, San Jose, CA) mass spectrometer, Hewlett Packard 1100 binary HPLC pump, and a Keystone Scientific (Bellefonte, PA) Betasil C18 column (100 × 2 mm). The in vitro assay mixtures were sampled directly by injecting 20 µl for analysis. Column flow (0.5 ml min^{-1}) was a linear gradient of methanol and 1% acetic acid. The gradient started at 10% methanol and was increased to 100% methanol over 20 min. Column flow was coupled to the LCQ mass spectrometer through an atmospheric pressure chemical ionization source (APCI) operating with a vaporizer temperature of 450°C, a sheath gas (N_2) flow of 70 units, an auxillary gas (N_2) flow of 10 units, a corona discharge current of 5 µA, a capillary tube temperature of 150°C, and a capillary voltage of −30 V. The mass spectrometer was set to scan a mass range of 100–1000 amu in the negative ion mode.

To calculate relative change in each dimer product, the peak areas from the LC–MS extracted ion chromatogram (m/z = 357 and 375) were first normalized

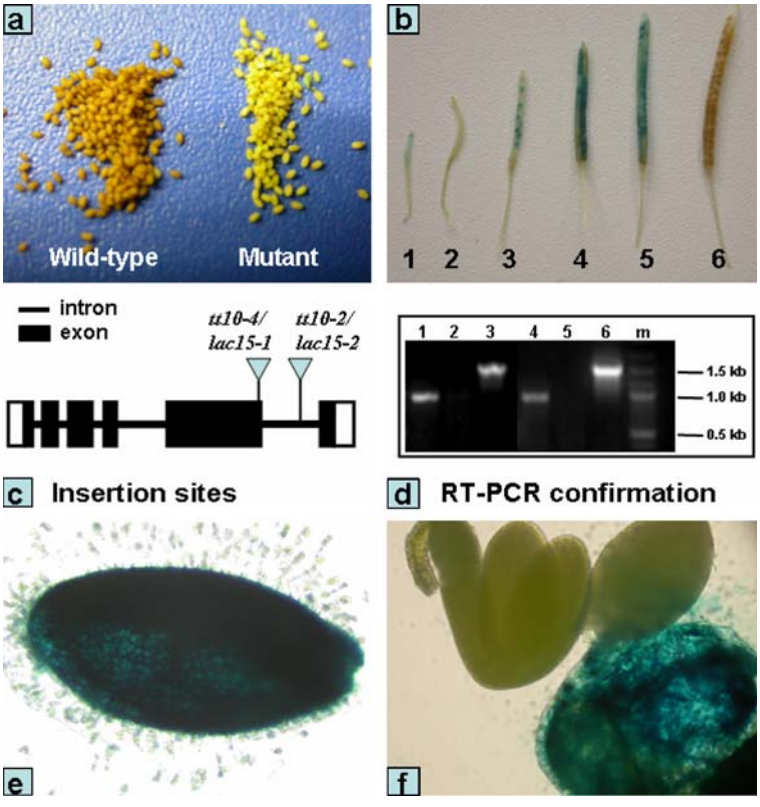

Fig. 1 Identification of *tt10/lac15* mutants and expression of *AtLAC15* in seed coats. (**a**) Color difference in newly matured wild-type (*left*) and mutant (*right*) seeds. (**b**) Expression of a GUS reporter gene driven by the *AtLAC15* promoter during seed development. Stages 1–5 correspond to the siliques with lengths of 0–3, 3–6, 6–9, 9–12 and >12 mm, respectively. Stage 6 represents the newly matured siliques (*brown*). (**c**) Mutant *tt10-4/lac15-1* (SALK_002972) has an insertion in the end of the fifth exon, and *tt10-2/lac15-2* (SALK_128292) has a T-DNA insertion in the fifth intron. (**d**) RT-PCR analysis shows *AtLAC15* transcripts in wild-type plants but not in the mutant plants. All cDNA samples showed an actin gene band as a quality control (data not shown). Lanes 1 and 4: presence of transcripts in wild-type plants; lanes 2 and 5: lack of transcripts in *tt10-4/lac15-1* and *tt10-2/lac15-2*, respectively; lanes 3 and 6: PCR products using genomic DNA as a template and using the same primer pair used for RT-PCR reactions in lanes 1, 2, 4 and 5. The size of PCR products in lanes 3 and 6 is greater due to the presence of an intron. (**e**) Expression of a GUS gene driven by the *AtLAC15* promoter in the whole seed from a stage-4 silique. (*f*) Seed coat, but not the embryo (green), was stained with GUS. Embryos and seed coats were separated first and then used for the GUS assay

by seed fresh weight of each sample and then divided by the normalized total peak area of all the monomers and dimers as a percentage. A small amount of dimers were detected in the commercial substrate and was subtracted during data calculations.

Root elongation analysis

To examine the effect of a mutation in *AtLAC15* on root elongation, seedlings were grown in a square Petri dish ($10 \times 10 \times 1.5$ cm^3) containing 35 ml of sterile solid medium consisting of 0.5 x MS salt, 0.5% sucrose, 10 mM Mes and 0.6% Phytagel (Sigma, St Louis, MO) at pH 5.8. Freshly harvested seeds were first surface sterilized and arranged on the surface of the solid medium and given a cold treatment at 4°C for 72 h. Ten wild-type and 10 mutant seeds were germinated on the same plate and plants were grown on a light shelf under the conditions described above. The plates were placed vertically on a rack so that the roots grew downward on the surface of the growth medium. Root growth was tracked for 5 days by marking the position of root tips on the plate over time.

Tetrazolium assay for seed coat permeability

The seed coat permeability assay was conducted by following the method described by Debeaujon et al. (2000). Briefly, about 30 freshly harvested mature seeds were incubated in a 1% (w/v) aqueous solution of tetrazolium (2,3,5-triphenyltetrazolium chloride) at 30°C in the dark for 1–5 days. Pink-stained seeds (at least half of the seed became pink) were counted daily for 5 days under a dissecting microscope. The experiment was conducted three times using independent seed batches.

Seed germination assay

Mutant or wild-type seeds (~100 each) were sown directly in a well-watered potting mix (Enriched Potting Mix, Miracle-Gro Lawn Products Inc., Marysville, OH), and kept in a cold room (4°C) for 3 days. Seeds were germinated and seedlings were grown on a light shelf under a 14/10 h light/dark photoperiod. Seedlings were scored 2 days after the seeds were moved out of a cold room.

Results

Mutations in *AtLAC15* altered the seed coat color

We identified two T-DNA insertional mutants in the SALK collections (Alonso et al. 2003), SALK_002972 (*lac15-1*) and SALK_128292 (*lac15-2*), with altered seed color (Fig. 1a). Seeds from the homozygous mutant plants were golden yellow whereas seeds from wild-type or heterozygous plants were dark brown for newly matured seeds. However, the color of the mutant seeds slowly changed to pale brown, becoming only slightly lighter than the color of wild-type seeds after 2 months or longer storage. The phenotype resembled the *tt10* mutant (Shirley et al. 1995) whose locus was mapped to chromosome 5 (www.arabidopsis.org) near *AtLAC15* (At5g48100). A recent study confirmed that *lac15* is allelic to *tt10* (Pourcel et al. 2005). To be consistent with the study by Pourcel et al. (2005), we used *tt10-4* for *lac15-1* and *tt10-2* for *lac15-2* in this study.

Mutant *tt10-4* has an insertion in the end of the fifth exon or four base pairs from the fifth intron. Mutant *tt10-2* has a T-DNA insertion in the fifth intron (Fig. 1c). The insertion sites were confirmed by sequencing the PCR products from a reaction using LBb1 primer paired with a gene-specific primer. RT-PCR analysis confirmed that both mutants are true null mutants (Fig. 1d). A full recovery of seed color phenotype from yellow to normal brown was just reported in a complementation experiment (Pourcel et al. 2005).

AtLAC15 is expressed in the seed coat and developmentally controlled during seed maturation

The gene expression of *AtLAC15* was studied using a GUS reporter gene driven by *AtLAC15* promoter (P_{lac15}::GUS). The GUS staining was first observed in the aborted siliques (0–3 mm or stage 1 in Fig. 1b). The color was localized in the transmitting tissues in the style and replum of siliques (data not shown). No GUS staining was observed from normal developing siliques between 0 and 6 mm. The GUS staining appeared when siliques were about 6–9 mm and reached the maximum intensity when siliques were 9–15 mm (stages 4 and 5). Siliques showed minimal staining when the seed coats started turning brown (Fig. 1b). A closer examination demonstrated that the color was solely due to the staining of seeds as no staining was observed in the seed pods (e.g., see Fig. 1b, stage 3).

By separating seed coats from embryos, it was observed that GUS staining was specifically localized in the seed coat, not in the embryo (Fig. 1e, f). *AtLAC15* was also expressed in roots based on a RT-PCR analysis (Cai and Wu, unpublished data), which was supported by the microarray expression data in the *Arabidopsis* database (www.arabidopsis.org) and a recent study by McCaig et al. (2005). However, no

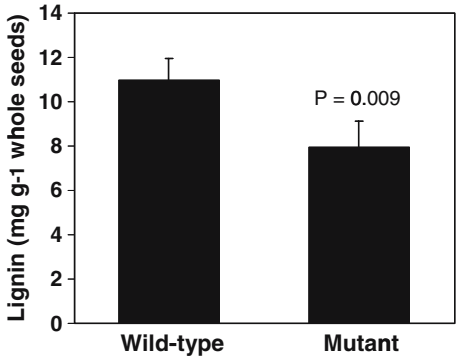

Fig. 2 Lignin content in wild-type and *tt10* mutant seeds. Thioglycolic lignin content was analyzed on whole seeds using a beech lignin as a standard (see Materials and methods). The data are means ± SE of four biological experiments with triplicate measurements in each experiment. The *P*-value is for a *t*-test for means of paired samples

GUS staining was observed in the roots, probably due to low gene expression. GUS staining was not observed in any other tissues examined.

Mutations in *AtLAC15* resulted in a reduction in lignin content and an accumulation of proanthocyanidin (PA) in seeds

Since seed coat color in *Arabidopsis* is believed to be determined mainly by PA and other flavonols (Shirley et al. 1992, 1995; Chapple et al. 1994; Debeaujon et al. 2001), we quantified the acetone-soluble PA. PA content measured with the butanol–HCl method (Mole and Waterman 1987b; Xie et al. 2003), however, was increased by 59% in the mature mutant seeds compared to the wild-type seeds (Supplement Fig. 1).

Laccases are believed to be involved in lignin synthesis based on their in vitro enzymatic activity of crosslinking monolignols, as described in the introduction. Also, a recent study demonstrated that thioglycolic lignin was significantly lower in yellow seeded-samples compared with the dark-seeded accessions in *Brassicaceae* (Marles and Gruber 2004). Thus we also examined the extractable lignin content in the mature mutant and wild-type seeds (Campbell and Ellis 1992; Marles and Gruber 2004). Mutant seeds showed near a 30% decrease in lignin content compared to wild-type seeds on the basis of seed weight (Fig. 2). Since there is no difference in seed weight and in cell wall dry weight between *tt10* and wild-type seeds (Supplement Table 1), a decrease in the lignin content in *tt10* will also be valid on the basis of seed number or cell wall dry weight.

Developing *tt10* seeds showed a reduced activity in monolignol polymerization

Lignin and PA synthesis are two of the major branches in the phenylpropanoid pathway. To exclude the possibility that a reduction in lignin content in *tt10* mutant seeds is due to an accumulation of PA or substrate competition, we directly examined the activity of polymerization of coniferyl alcohol in the developing mutant and wild-type seeds. At this stage (stage 4 in Fig. 1b), the outermost several cell layers of the young seeds which eventually are fused to form an amorphous and protective seed coat are actively metabolizing (Beeckman et al. 2000; Windsor et al. 2000). Based on the study by Pourcel et al. (2005), *TT10/AtLAC15* is expressed at the greatest level in the second outermost cell layer in these developing seeds. Thus, substrates should be easily accessible to those cell layers. Coniferyl alcohol is a major monomer that is incorporated into lignin in *Arabidopsis* (Dharmawardhana et al. 1992; Chapple et al. 1994). In this in vitro assay, green seeds were incubated with coniferyl alcohol substrate in the presence of catalase (to eliminate H_2O_2 and thus peroxidase activity) (Pourcel et al. 2005). Liquid Chromatography-Mass Spectrometry (LC–MS) was used to detect the products from the in vitro assay. Two typical chromatograms of the products from the reaction mixture of mutant and wild-type seeds are shown in Fig. 3 Peaks, C1 and C2, with the same mass spectrum as coniferyl alcohol standard ($m/z = 179$, M-1, relative intensity 20%; $m/z = 177$, M-3, base peak) were detected in both reaction mixtures of wild-type and mutant seeds as well as in a standard solution. C1 and C2, however, were not detected in the seed-only control, indicating no accumulation of endogenous substrates. Peak C1 was identified as coniferyl alcohol based on its retention time and that of the standard, whereas peak C2 is most likely the *cis* isomer. Consistently, the two monomer peaks were reduced in the reaction mixture of the wild-type seeds while several other peaks were enlarged compared to the reaction mixture of the mutant seeds. Among them (Fig. 3c, d), four peaks, D1–D4, showed mass spectra consistent with known coniferyl dimers (Boerjan et al. 2003). D1 and D4 are possible β-β and/or β-5 dimers ($m/z = 357$, M-1), and D2 and D3 are β-O-4 dimers ($m/z = 375$, M-1). Since β-5 and β-β dimers have the same molecular weight, the linkage in D1 and D4 cannot be determined based on the mass spectrum alone. Dimer formation was not detected in the reaction mixture of heat-killed seeds (one of the negative controls). As summarized in Fig. 4, the amount of three of these dimers (D1, D2 and D4) were significantly greater in

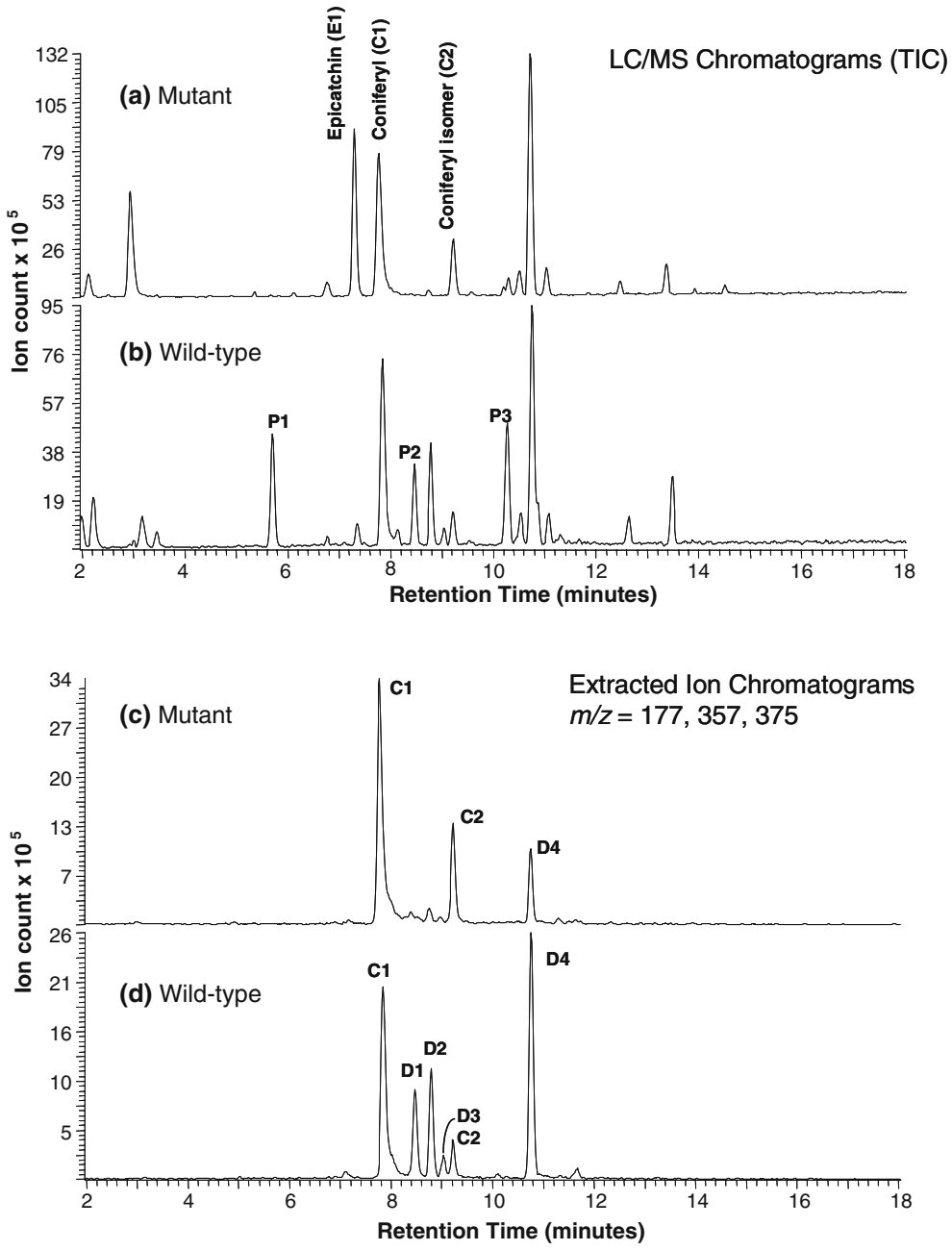

Fig. 3 LC/MS chromatograms of the reaction mixtures from an in vitro polymerization reaction using coniferyl alcohol and developing mutant or wild-type seeds. Total ion chromatograms of mutant (**a**) and wild-type (**b**) seed reactions. Coniferyl alcohol (C_1) present at retention time (R_t) = 7.90 and its isomer (C_2) R_t = 9.26. Epicatechin (*E1*) was observed at R_t = 7.38. P1, P2, and P3 (m/z = 401) are unidentified compounds which have a calculated molecular weight of 222 greater than coniferyl alcohol and were reproducibly more abundant in the wild-type seed reaction. Extracted ion chromatograms from LC/MS analysis of mutant (**c**) and wild-type (**d**) seed reactions are shown, illustrating coniferyl monomer and dimer components. Extracted ions m/z = 177 for monomer, 357 and 375 for dimers. C_1 coniferyl alcohol, C_2 coniferyl alcohol isomer, *D*1 and *D*4 β-β dimer and/or β-5 dimer, *D*2 and *D*3 β-O-4 dimer

the wild-type seed reaction compared to that in the mutant seed reaction, strongly indicating a reduction in laccase activity in mutant seeds. Also observed from the LC–MS analysis were several peaks, P1, P2 and P3 (m/z = 401) (Fig. 3b), that were reproducibly higher in the wild-type seed reaction. They had a calculated molecular weight of 222 greater than coniferyl alcohol. Since these compounds were not detected in the seed-only control (seeds without addition of coniferyl alcohol), they were potentially conjugates of coniferyl

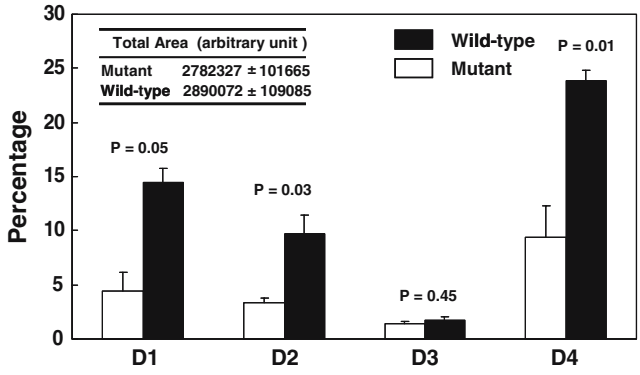

Fig. 4 Differences in each coniferyl dimer formed in an in vitro assay by incubating coniferyl alcohol with developing mutant or wild-type seeds. D1 and D4 = β-β dimer and/or β-5 dimer, D2 and D3 = β-O-4 dimer. No endogenous coniferyl alcohol monomers and dimers were detected from the seed-only control. A small amount of D4 dimers were detected in the standard and were subtracted from the total amount of D4 dimers during the calculation. Inset table shows no difference in the total peak area of all the monomers and dimers in the mutant and wild-type seed reactions ($P = 0.81$). The data are means ± SE of four independent experiments. The *P*-values are for a *t*-test for means of paired samples

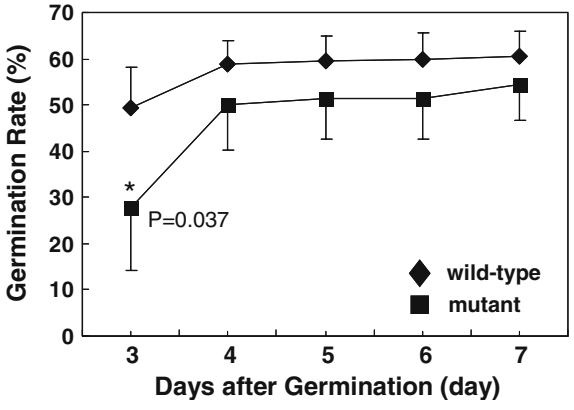

Fig. 5 Germination rate of wild-type and mutant seeds in soil. Seeds were directly sown in soil and kept at 4°C for 3 days. Seed germination rates were scored each day, starting from the third day after seeds were incubated at room temperature on a light shelf. The data are means ± SE of three experiments with $n \approx 100$ in each experiment. The asterisk indicates statistical difference ($P = 0.037$) from a *t*-test for means of paired samples

alcohol with an unknown compound. No trimers or other polymers were observed in any reactions. Interestingly, we repeatedly observed a significantly larger peak, E1, in the mutant seed reaction mixture than the wild-type seed reaction. The mass spectrum and retention time of the peak corresponded to (–)-epicatechin standard (ChromaDex, Santa Ana, CA). The results indicated that epicatechin was released from the developing seeds and that mutant seeds may accumulate more epicatechin than wild-type seeds.

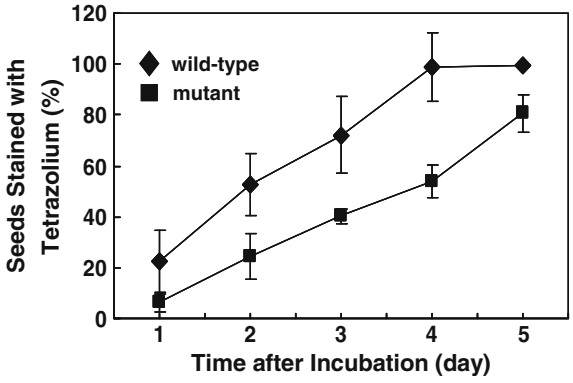

Fig. 6 Seed coat permeability to tetrazolium. About 30–50 seeds per line were incubated in a 1% (w/v) aqueous solution of tetrazolium at 30°C in the dark for one to 5 days. Pink-stained seeds (at least half of the seeds became pink) were counted daily for 5 days under a dissecting microscope. The experiment was conducted three times using independent seed batches. Error bars not shown are hidden by the symbols

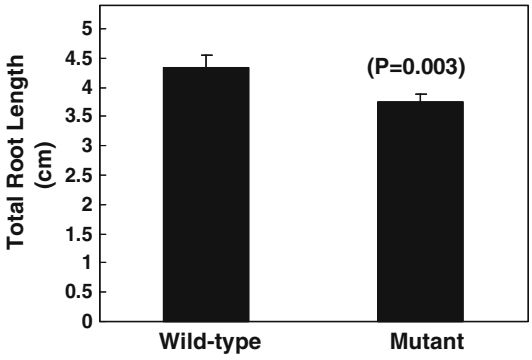

Fig. 7 Primary root elongation in *tt10* and wild-type seedlings. Ten seeds each for both wild type and mutants were germinated on the same plate and plants were grown on a light shelf under the conditions described in Materials and methods. The plates were placed vertically on a rack so that the roots grew downward on the surface of the growth medium. Root growth was tracked by marking the position of root tips on the plate with time. The experiment was conducted three times with two technical replicates in each experiment. The *P*-value is for a *t*-test for means of paired samples

tt10 showed various altered physiological features

We examined whether the change in PA and lignin content in the mutant seeds would affect seed physiology. As shown in Fig. 5, mutant seeds showed a consistent decrease in germination rate in soil compared to wild-type seeds. However, due to large variation among different experiments, a difference in germination rate was significant only on the third day after germination. When germinated on MS-salt-Phytagel, both mutant and wild-type seeds showed almost 100% germination (data not shown).

Due to the nature of PA and lignin polymers, change in composition of PA and lignin in the mature

seed coat can potentially affect seed coat physical properties. Previously, it was reported that the seed coat permeability in some PA mutants, such as *transparent testa 1, 10 and 13*, was affected based on the permeability to tetrazolium salt (Debeaujon et al. 2000). Tetrazolium salt will be reduced and produce a pink-red color after the salt penetrates the seed coat and reaches the embryo. Stained mature wild-type seeds were dark red and the stained mature mutant seeds were only pinkish (Supplement Fig. 2). In addition, the wild-type seeds were stained faster than the mutant seeds during a time-course experiment (Fig. 6), indicating the salt diffused through the wild-type seed coat more freely. Mutant seeds reached nearly a 100% staining after a 5-day incubation.

AtLAC15 is weakly expressed in the roots based on RT-PCR analysis (McCaig et al. 2005). An examination of root elongation for 5 days showed the roots of *tt10* elongated slower than wild-type roots (Fig. 7) on MS-salt-phytagel plates.

Discussion

Regulation of gene expression of *AtLAC15*

AtLAC15 is one of the 17 annotated laccase or laccase-like genes in *Arabidopsis* and resembles other known plant laccases (McCaig et al. 2005; Pourcel et al. 2005). Based on the gene expression analysis using GUS as a reporter gene, *AtLAC15* was specifically expressed in the seed coat and highly expressed during the late stage of seed development (Fig. 1b, e, f). Our results are in agreement with the findings in a recent study by Pourcel et al. (2005) who also showed that the expression of *AtLAC15* was controlled in a temporal and spatial manner. The gene expression pattern and location is consistent with the potential role of *AtLAC15* in deposition of PA and lignin in the seed coat (see Discussion below).

Our unpublished results using RT-PCR (Cai and Wu, unpublished data) showed that *AtLAC15* was also weakly expressed in roots, which confirmed the data from the *Arabidopsis* microarray database and a recent study by McCaig et al. (2005). However, the GUS staining was not observed in roots in our assay, presumably due to its low expression in the roots. *AtLAC15* was also reported to be expressed in leaves and stems (Pourcel et al. 2005) which were not confirmed by our GUS staining. Again a low gene expression level as well as variations in plant culture conditions may have contributed to the difference in results.

Two independent insertions of T-DNA in *AtLAC15* resulted in the same pale seed color phenotype, strongly suggesting that the phenotype was due to the mutation in this specific gene. Also, the mutant phenotype was fully reversed in a complementation experiment (Pourcel et al., 2005).

Mutant *tt10* seeds accumulate more soluble PA than wild-type seeds

Seed color in *Arabidopsis* is mainly determined by the content of flavonols and PA metabolites in the seed coat (Shirley et al. 1992, 1995; Chapple et al. 1994; Debeaujon et al. 2001). An altered seed coat color in *tt10* prompted a measurement of PA content in the seeds. Different from most of the other seed color mutants (Debeaujon et al. 2003), *tt10* mutants showed an increase in the acetone-soluble PA content in the seeds, suggesting the brown seed coat color in wild-type seeds is mainly due to further oxidation of PA products. PAs are colorless compounds and need to be further oxidized presumably by peroxidases or polyphenol oxidases into brown products, which contribute to the brown seed coat color (Debeaujon et al. 2000, 2003; Kitamura et al. 2004). The yellow seed coat we observed in *tt10* mutants suggests that *AtLAC15* potentially acts at the oxidation step to convert PA into an oxidized form. Our results are similar to the findings by Pourcel et al. (2005) who demonstrated that the mutant seeds showed an increase in total PA content. In addition, Pourcel et al. (2005) showed more than a 4-fold increase in epicatechin content in the mature mutant seeds, indicating an accumulation of the precursors for PA synthesis. Our LC–MS analysis (Fig. 3) demonstrated that the mixture of developing mutant seed reaction showed a greater peak for epicatechin compared to the mixture of wild-type seed reaction, supporting their observations. In accordance, Pourcel et al. (2005) also demonstrated that the developing wild-type seeds showed a greater activity of polymerization of epicatechin in vitro than mutant seeds. Although the interflavan linkages of the products in their in vitro assay were different from the ones in natural PA, their results strongly suggest that *AtLAC15* may perform the same function, i.e. PA synthesis, in vivo. Thus, the normal function of *AtLAC15* may be involved in polymerization of PA into larger polymers and probably further oxidation of PA, which may confer brown color to the seed coat (Pourcel et al., 2005).

Mutation of *AtLAC15* led to a reduction in lignin content in mature seeds and in activity of monolignol polymerization in developing seeds

Laccases in plants are able to polymerize lignin monomers in in vitro assays (Sterjiades et al. 1992; Bao et al.

1993). However, genetic evidence for the role of laccase in lignin synthesis in vivo is still lacking. Analysis of *tt10* seeds showed a 30% decrease in extractable lignin content. According to a comparative measurement from seed coats and embryos, Marles and Gruber (2004) showed that the majority of the seed lignin was present in the seed coat and that yellow seed accessions in the *Brassicaceae* had significantly lower lignin content than seeds of the brown/dark-seeded accessions. Hence, based on its predominate expression pattern in developing seed coat, *AtLAC15* may control lignin synthesis in *Arabidopsis* seed coats. Since lignin and PA synthesis are two of the branches in the phenylpropanoid metabolism pathway (Croteau et al. 2000) and share a common precursor, phenylalanine, a decrease in lignin synthesis could potentially lead to a reallocation of substrates to other branches in the pathway, such as providing more precursors for PA synthesis. This could result in the accumulation of PA that was observed. Alternatively, a decrease in lignin content in mutant seeds could be due to a high substrate demand for increased PA synthesis as substrate competition. However, the difference in in vitro polymerization activity of epicatechin (Pourcel et al. 2005) or coniferyl alcohol in this study between developing mutant and wild-type seeds suggest that *AtLAC15* could be involved in both PA and lignin synthesis directly.

We were only able to detect coniferyl dimers from the in vitro laccase activity assay. The dimers formed included the β-O-4 linkage and β-5 and/or β-β linkages. The amount of β-O-4 linkages (D2 and D3 in Figs. 3, 4) among the total dimers (sum of D1–D4) formed in the in vitro assay is about 23% for wild-type seeds and 25% for mutant seeds, which is significantly lower than that in native lignin of xylem or stem tissues of other plants. A higher percentage of β-5 and β-β linkages than the β-O-4 linkage has been observed often in in vitro monolignol polymerization reactions (reviewed in Croteau et al. 2000; Boerjan et al. 2003). Many factors in the in vitro assay could affect the formation of different linkages. The concentration of monomers, pH, or the presence of cell wall components could affect the distribution of different linkages (Terashima et al. 1996). Dirigent proteins have been shown to control the stereo-specificity of linkages during lignan synthesis and have also been proposed to be involved in lignin assembly (Davin and Lewis 2005). This could explain the major difference in linkages between native lignin and the products from in vitro reactions. The reason why we did not detect trimers or polymers in the in vitro assay is not clear. It is possible that the amount of trimers or polymers formed is too little to be detected due to the factors mentioned above for in vitro reactions. Alternatively, the polymerization of lignin involving laccase in vivo could require other components in cell walls, which would lead to a direct incorporation of monomers into the seed coat and leave no polymer products in the solution. Of course, the possibility that *AtLAC15* is also involved in lignan synthesis in vivo should not be excluded. The mechanism of lignin formation from monomers is still not clear and is a field of active research (reviewed in Croteau et al. 2000; Boerjan et al. 2003). Further studies with this laccase mutant will provide valuable information about lignin synthesis *in planta*.

Physiological roles of *AtLAC15*

PA in the seed coat plays important roles in seed physiology, such as a protective role in biotic and abiotic stresses (reviewed in Dixon et al. 2005), partially through regulating seed coat physical properties. Previously, a decrease in PA content in many other *transparent testa* mutants was associated with an increase in seed coat permeability to salt (Debeaujon et al. 2000). In contrast, *tt10* mature seeds showed a slow permeability to tetrazolium salt (Fig. 6). A 59% increase in PA content in the mutant seed coat may have decreased the pore sizes in the seed coat, reducing the permeability of the mature seed coat. It bears mentioning that a similar permeability assay with *tt* mutant seeds showed little color staining in the *tt10* seeds and wild-type seeds of different ecotypes (Debeaujon et al. 2000). This discrepancy with our results is probably due to differences in plant culture protocols and the time frame for harvesting seeds in the permeability test. In addition, Debeaujon et al. (2000) examined their stain accumulation (in their permeability test) within 2 days. In our test, this is not a stage that shows significantly different permeability between *tt10* and wild-type seeds. Lignification of cell walls is also known to affect permeability to water and solutes in other tissues (Croteau et al. 2000). However, it is not clear how much of an impact the reduction in lignin content in *tt10* had on the seed coat permeability.

The altered seed coat properties in *tt10* may have contributed to delayed germination (Fig. 5) in soil on the third day compared to wild-type seeds. However, the germination of *tt10* seeds was not significantly delayed on the MS-salt-Phytagel plates compared to the wild-type seeds (data not shown), which is consistent with an earlier study in which the germination of *tt10* seeds on filter papers was not reduced compared to wild-type seeds (Debeaujon et al. 2000). Thus, a decrease in germination rate for *tt10* seeds in soil on

the third day may be due to an altered sensitivity to unfavorable growth conditions.

AtLAC15 is expressed in the roots (McCaig et al. 2005), although at a very low level (www.arabidopsis.org) (Cai and Wu, unpublished data). Root elongation of *tt10* was significantly reduced compared to wild-type seedlings (Fig. 7), suggesting that *AtLAC15* is required for normal root growth. The question is whether *AtLAC15* is involved in the same biochemical process as it is in the seed coat, i.e. lignin and PA metabolism. Since the development of root xylem and the Casparian strip requires deposition of lignin, future studies should include examination of xylem and Casparian strip structure and development.

In summary, we have demonstrated that a mutation in *AtLAC15* affected both extractable lignin and soluble PA content in *Arabidopsis* seeds. Our studies showed that developing *tt10* seeds reduced the in vitro polymerization activity of coniferyl alcohol compared to wild-type seeds, suggesting that *AtLAC15* could perform the same function in vivo. To our knowledge, this is the first genetic evidence for the involvement of a laccase in lignin synthesis. Together with a recent study (Pourcel et al. 2005), which showed a role of *AtLAC15* in the *in vitro* polymerization of epicatechin, the findings from *tt10* mutant studies strongly indicate the dual role of *AtLAC15* in PA and lignin synthesis in *Arabidopsis* seeds.

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